VERSITA

Cent. Eur. J. Chem. • 11(1) • 2013 • 94-100
DOI: 10.2478/s11532-012-0134-y

Central European Journal of **Chemistry**

Drug screening in human plasma by cloud-point extraction and HPLC

Research Article

Katarzyna Madej*, Karolina Persona

Department of Analytical Chemistry, Faculty of Chemistry, Jagiellonian University, 30-060 Krakow, Poland

Received 20 July 2012; Accepted 8 September 2012

Abstract: Cloud-point extraction (CPE) with RP-HPLC/DAD detection was used to develop a screen for six model basic drugs (paracetamol, promazine, amitriptyline, nortriptyline, clomipramine and chlorpromazine) in human plasma. These drugs' varied hydrophobicities entail different affinities for the micelle-rich phase and CPE extraction efficiencies. Extraction recovery (except paracetamol) was above 80% and reproducibility (RSD%) ranged from 2.88 to 10.26 intraday and from 3.12 to 12.33 interday. The limits of detection were: 0.125 μg mL^{-1} (promazine and chlorpromazine), 0.25 μg mL^{-1} (amitriptyline and nortriptyline) and 0.5 μg mL^{-1} (paracetamol and clomipramine). The method was linear over the ranges: 0.125 -1.0 μg mL^{-1} (promazine and chlorpromazine), 0.25-1.0 μg mL^{-1} (amitriptyline and nortriptyline), 0.5-1.0 μg mL^{-1} (clomipramine) and 0.5-10 μg mL^{-1} (paracetamol). The procedure is a good alternative to the SPE or LLE sample preparation usually used.

Keywords: *Cloud-point extraction • Micelle-mediated extraction • Screening HPLC analysis • Basic drugs*

1. Introduction

Screening of body fluids - usually urine, plasma/serum or whole blood - is performed in overdosing on unknown drugs [1]. In some emergencies quick and automated immunoassays which do not require special sample preparation are used. However, these only determine some drug groups (*e.g.* benzodiazepines, tricyclic antidepressants) and confirmation should be performed by HPLC or capillary electrophoresis (CE) [1]. Despite advances in instrumental techniques, drug determinations in biological samples are usually preceded by pretreatment to isolate and concentrate the drugs; chromatographic and electrophoretic instruments cannot handle the matrix. Classic liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are most commonly employed [1].

Paracetamol, amitriptyline, nortriptyline, promazine, chlorpromazine and clomipramine are also usually isolated from biological material using SPE or LLE, and determined by HPLC/UV(DAD). Amitriptyline, nortriptyline and clomipramine have been extracted from human plasma on Isolute C2 cartridges with recoveries of 81±3.1%, 95±5.0% and 101±6.2%, respectively [2], then determined together with other tricyclic antidepressants and their metabolites by HPLC/UV. In combination with SPE an HPLC/UV method using Isolute cyanopropyl (CN) cartridges [3] was applied to simultaneous analysis of classical neuroleptics (including chlorpromazine), atypical neuroleptics, and their metabolites in human plasma. Extraction yields were all ≥ 93%. Five antipsychotic drugs, including promazine, were simultaneously determined in rat plasma by HPLC/UV after an LLE acid back extraction using isopropyl ether-pentane [4]. The extraction yield for promazine was *ca*. 100% with intra-day and inter-day precisions (RSD) *ca*. 3.6% and 4.3%. Liquid-liquid extraction with 2-propanol-chloroform was also successfully employed in isolation and determination of paracetamol and its toxic metabolite *N*-acetyl-*p*-benzoquinoneimine in pediatric plasma [5]. Along with the advantages of long experience, good recovery and repeatability, these techniques also require substantial time, consume large amounts of toxic organic solvents, or require relatively expensive extraction columns.

* *E-mail: madejk@chemia.uj.edu.pl*

Micelle-mediated extraction (MME), especially cloud-point extraction (CPE), seems to be a good alternative to conventional LLE and SPE. Its chief advantages are simplicity, speed, low cost, and compatibility with common HPLC mobile phases and CE background electrolytes, as well as the environmentally benign character of the surfactants.

In CPE a micellar phase is formed from a homogenous surfactant solution added to the sample. Surfactants aggregate into micelles, orienting their hydrocarbon tails toward the center creating a non-polar core. Hydrophobic compounds (most drugs) are favorably partitioned into that hydrophobic core. Phase separation into a micelle-rich phase and an aqueous phase containing surfactant near the critical micelle concentration requires a temperature change for non-ionic and zwitterionic surfactants (CPE). Changing other parameters (*e.g.* pH, ionic salt or organic solvent addition) yields phase separations with ionic surfactants [6].

A number of papers concerning drug determination using CPE (usually a single drug) in human urine [7-11], human serum [12-14] and rat plasma [15-17] have appeared in the last decade. In most reports, liquid chromatography with fluorimetric detection or UV detection above 280 nm was used. GC-MS has also been used, but additional cleanup was required [13]. To our knowledge, only paracetamol and chlorpromazine among the six drugs investigated here have been concentrated from body fluids by CPE. Paracetamol in urine was determined as the blue derivative of its hydrolysis product with tetrahydroxycalic[4]arene by conventional spectrophotometry after CPE with Triton X-114 [10]. For spiked and real urine samples the recoveries were 100±3%. The phenothiazines pericyazine, chlorpromazine and fluphenazine were isolated from spiked human serum and separated from the Triton X-114 by passing the surfactant–rich phase through a cation exchange column permitting determination by GC-FID [13]. The recoveries of pericyazine, chlorpromazine and fluphenazine were 95.1±3.3%, 87.1±6.3% and 84.7±2.7%, respectively.

The objective of this work was to test cloud-point extraction for toxicologic screening of basic drugs in human plasma. Six drugs: paracetamol, promazine, chlorpromazine, nortriptyline, amitriptyline and clomipramine were selected, representing a large group of basic drugs (pK_a > 9) with a wide range of hydrophobicity. The procedure was applied to HPLC-DAD screening for these compounds in plasma, and appropriate validation parameters were determined. The drug structures, molecular weights, dissociation constants (pK_a) and octanol-water partition coefficients (logP) are in Table 1.

2. Experimental procedure

2.1. Apparatus and chromatographic conditions

A Merck-Hitachi (Darmastadt, Germany) LaChrom chromatographic system, consisting of an L-7100 pump and an L-7455 programmable diode array detector (DAD) was used with a Merck Nucleosil C8 column (250×4.6 mm i.d., 5µm) thermostated at 25ºC. A Vibra Cell ultrasonic bath and FreeZone 11 lyophilizer were from Sonics & Materials Inc. (USA) and Labconco (USA), respectively. The samples were centrifuged using an MPW-6 ultracentrifuge (Mechanika Precyzyjna, Poland).

Gradient chromatography used mobile phase A: 0.002M aqueous orthophosphoric acid, and phase B: acetonitrile. The gradient profile was: 0 min: 100% A, 0-30 min: 30% A and 70% B and 30-33 min: 100% A. The flow rate was 1mL min^{-1}. The drugs were detected by UV absorption at 254 nm.

2.2. Reagents

HPLC-gradient grade acetonitrile and methanol were supplied by Merck (Germany) and Triton X-114 were purchased from Sigma-Aldrich (Germany). The 85% orthophosphoric acid, 30% sodium hydroxide, and 25% ammonia, all of analytical grade, were purchased from POCH (Poland). Doubly deionized water (<1.0 µS cm^{-1}) was used throughout.

2.3. Drugs and Materials

Amitriptyline, nortriptyline, clomipramine and paracetamol were purchased from Sigma-Aldrich (Germany), and promazine and chlorpromazine were obtained from the Jelfa pharmaceutical factory (Poland). A stock solution of each drug (10 mg mL^{-1}) was prepared in methanol and stored at 4ºC. Working drug solutions were prepared by dilution of the stock drug solutions with a 1:1 (v/v) mixture of phases A and B. Human plasma was obtained from the Krakow, Poland blood bank. This plasma was spiked with water-diluted standard drugs to obtain the control samples.

2.4. Sample Preparation

Plasma (1 mL) was mixed with 1 mL of 7.5% (w/v) Triton X-114, adjusted to pH 12 and incubated at 25°C for 20 min. Centrifugation (13,000 rpm for 10 min) was performed and the sample was cooled in an ice bath for 5 min. The upper aqueous phase was decanted and the

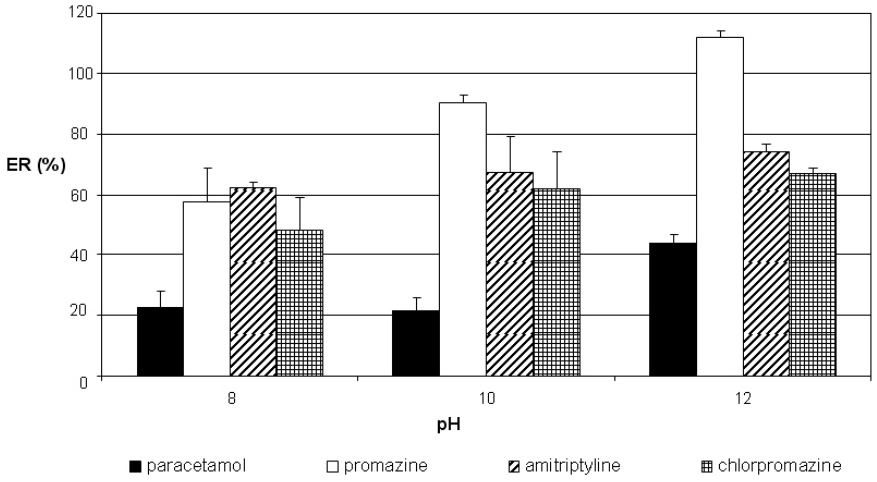


Figure 1. Effect of sample pH on extraction recovery from aqueous standard solutions (each drug at 1 μg mL^{-1}).

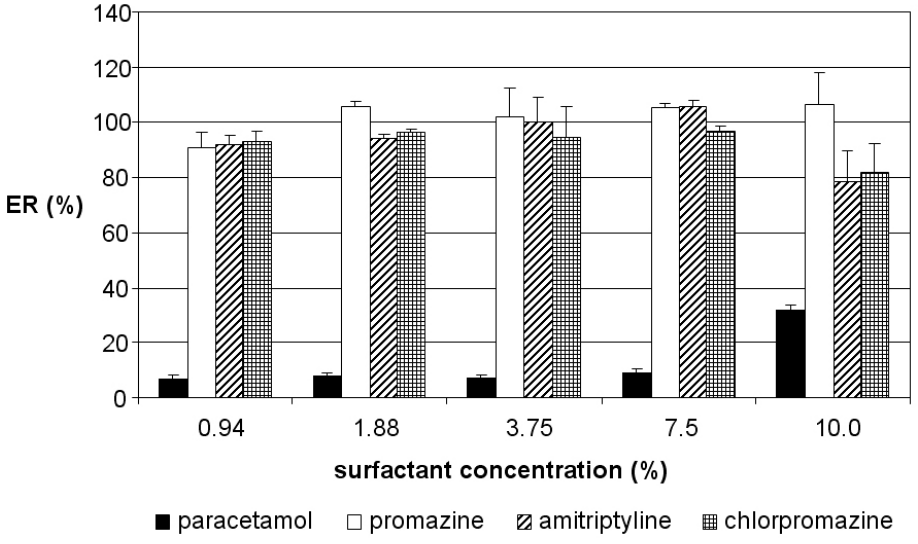


Figure 2. Effect of surfactant concentration (Triton X-114) on extraction recovery from aqueous standard solutions (each drug at 1 μg mL^{-1}).

micelle-rich phase containing the drugs was evaporated under a nitrogen stream in a hot block at 40°C for 30 min. The dried micellar phase was dissolved in 150 µL of acetonitrile.

3. Results and discussion

Paracetamol, promazine, amitriptyline and chlorpromazine were used to optimize the CPE conditions. Sample pH, surfactant concentration, centrifugation speed and time, as well as the micellar phase evaporation method were optimized. However, incubation temperature (25°C), incubation time (20 min), and acetonitrile volume (150 µL) were based on previous work [18]. The degree of drug recovery was the main optimization criterion.

3.1. Study of CPE conditions

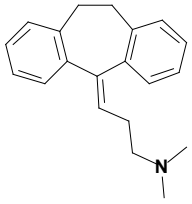
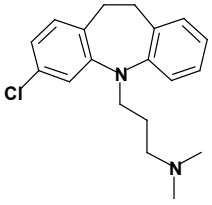
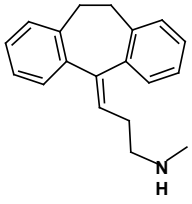
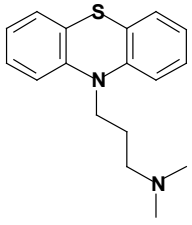
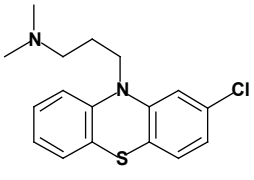
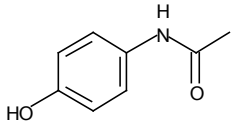
3.1.1. Effect of sample pH

The effect of sample pH was examined applying the above procedure using 7.5% (w/v) Triton X-114 at pH 8.0, 10.0 and 12.0. The average extraction recoveries (4 replicates) are in Fig. 1, and the optimal sample pH 12 was used in further experiments.

3.1.2. Effect of surfactant concentration

Surfactant concentration is a compromise between preconcentration and a resulting surfactant-rich phase volume sufficient to make reproducible extractions. Drug recovery was examined at five surfactant concentration levels: 0.94, 1.88, 3.75, 7.5 and 10.0% (w/v). The average extraction recoveries (4 replicates) are in Fig. 2. From 1.88 to 7.5% the extraction recovery for promazine, amitriptyline and chlorpromazine was

Table 1. Structure, molecular mass, dissociation constant and octanol-water partition coefficient.

Drug	Structural formula	Molecular mass [g mol^{-1}]	pK_a (25°C)	LogP[b]
Tricyclic antidepressants				
Amitriptyline		277.4 (313.9)[a]	9.4	4.9
Clomipramine		314.9 (351.3)[a]	-[c]	5.2
Nortriptyline		263.4 (299.8)[a]	9.7	1.7
The derivates of phenothiazine				
Promazine		284.4 (320.9)[a]	9.4	2.5
Chlorpromazine		318.9 (355.3)[a]	9.3	3.4
The derivative of p-aminophenol				
Paracetamol		151.2	9.5	0.5

[a]) *molecular mass of hydrochloride salt*
[b]) *participation coefficient (octanol/buffer 7.4)*
[c]) *lack of literature data*

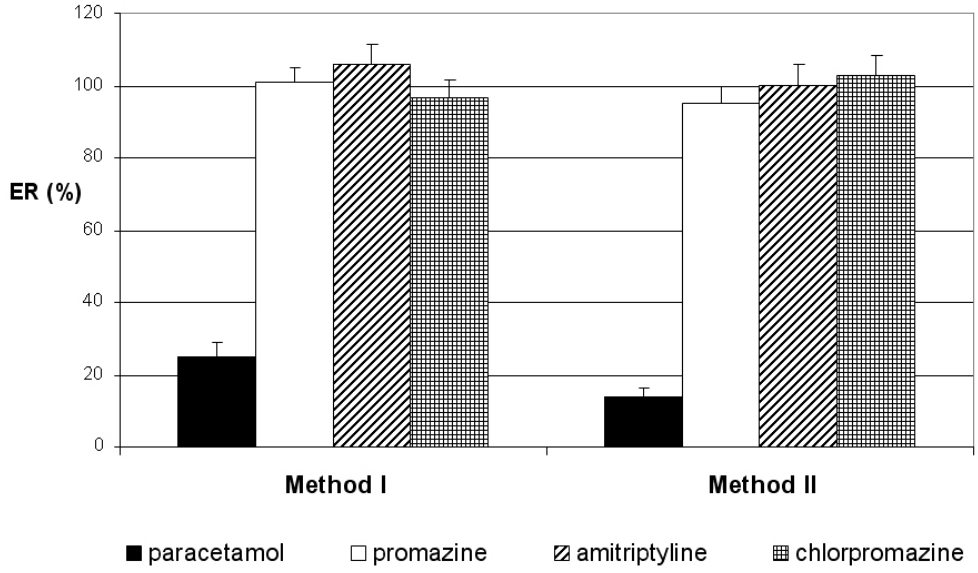


Figure 3. Effect of the micelle-rich phase treatment method. Method I: decantation of the upper aqueous phase and evaporation of the micellar phase at 40ºC in flowing nitrogen for 30 min. Method II: decantation of the upper aqueous phase and lyophilization of the micellar phase for 24 hours.

high and ranged from above 80 to *ca.* 100%. However, for the less hydrophobic paracetamol (smallest logP) the extraction recovery was < 10%. It was better extracted at considerably higher surfactant levels; *ca*. 30% recovery was achieved using 10.0% Triton X-114, while at this surfactant concentration the recoveries of the rest (except promazine; logP = 2.5) were lower (Fig. 2). Considering the relatively high concentrations of paracetamol in plasma and whole blood (therapeutic plasma concentrations range 10-20 μg mL^{-1}), the recovery (*ca*. 20 %) was sufficient for screening. Finally, 1 mL of 7.5% (w/v) Triton X-114 was selected.

3.1.3. Effect of centrifugation

The influence of centrifugation speed and time were examined. Three speeds (4500 rpm, 8000 rpm and 13000 rpm) and three times (5, 10 and 15 min) were tested. Higher centrifugation speed (especially 13000 rpm) gave better micellar phase adhesion to the tube walls. The centrifuge did not allow higher speed. Centrifugation time was of less significance. As the final centrifugation parameters 13000 rpm and 10 min were chosen.

3.1.4. Methods for micelle-rich phase quantitative evaporation

Handling the micellar phase containing the isolated drugs is critical for CPE reproducibility. Complete removal of water traces is usually by evaporation under nitrogen flow [6].

Two methods for quantitative removal of water from the micelle-rich phase after decantation of the upper aqueous phase were examined:

1) evaporation under nitrogen flow using a hot block (25ºC) for 20 min.

2) lyophilization for *ca*. 24 hours.

Using conventional heating incomplete decantation and dehydration caused scatter in the results. To improve reproducibility and improve the chromatograms for method (1) (separation quality strongly depended on quantitative water removal) the temperature was raised from 25 to 40ºC and the drying time increased from 20 to 30 min.

Lyophilization was performed in liquid nitrogen at -50ºC. The lyophilates were dissolved in acetonitrile (150 μL) in an ultrasonic bath for 10 minutes then analyzed.

The mean recoveries (4 replicates) for paracetamol, promazine, amitriptyline and chlorpromazine are in Fig. 3. Lyophilization gave recoveries similar to those from conventional heating with evaporation under nitrogen. Considering the time required for lyophilization the first method was chosen.

3.2. Validation of the CPE-HPLC/DAD method for HPLC determination of six basic drugs in plasma samples

The CPE procedure was applied to the six basic drugs paracetamol, amitriptyline, nortriptyline, promazine, chlorpromazine and clomipramine in human plasma. Example chromatograms of a standard drug mixture,

Table 2. CPE-HPLC/DAD method validation parameters for determination of the six basic drugs in plasma.

Parameter	Plasma					
	Paracet	Promaz	Nortript	Amitript	Chlorpro	Clomipra
Retention time (RT) [min]	7.09	17.40	18.01	18.50	19.29	20.11
Intraday RT precision [RSD, %] (n=4)	0.003	0.008	0.005	0.006	0.008	0.013
Interday RT precision [RSD, %] (n=4)	0.010	0.013	0.015	0.072	0.077	0.086
Extraction recovery (ER) [%]	22.08	94.11	103.66	91.98	86.28	82.49
Intraday ER precision [RSD, %] (n=4)	10.26	4.24	2.88	4.40	4.72	7.50
Interday ER precision [RSD, %] (n=4)	12.33	4.95	3.12	5.21	5.25	8.78
Linearity range [μg mL^{-1}]	0.5-10.0	0.125-1.0	0.25-1.0	0.25-1.0	0.125-1.0	0.5-1.0
Correlation coefficient r²	0.9976	0.9951	0.9974	0.9896	0.9989	0.9974
Detection limit (LOD) [μg mL^{-1}]	0.5	0.125	0.25	0.25	0.125	0.5

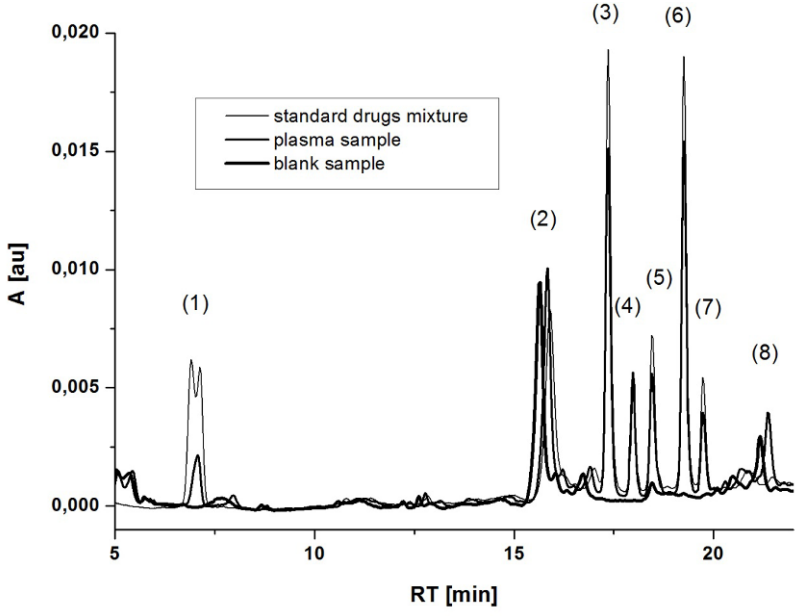


Figure 4. Post-CPE chromatograms of a standard drug mixture, a blank plasma sample, and a plasma sample spiked with the six tested drugs at 1μg mL^{-1}: 1– paracetamol, 3 – promazine, 4 – nortriptyline, 5 – amitriptyline, 6 – chlorpromazine, 7 – clomipramine, 2 and 8 – unidentified.

a plasma extract containing the six drugs, and a blank plasma extract after optimized CPE are in Fig. 4.

The extraction recoveries, reproducibility (intraday and interday), detection limit and linearity ranges are in Table 2. The retention times and their intraday and interday precisions were also determined.

The experimental conditions yield good separation for all the drugs examined and the Triton X-114. The plasma matrix does not interfere with analyte peaks (Fig. 4), so the selectivity is satisfactory.

To determine drug recovery the plasma was spiked with paracetamol, promazine, amitriptyline, chlorpromazine, nortriptyline and clomipramine at 1 µg mL^{-1} each, then submitted to CPE-HPLC. Extraction recovery was determined from the peak

area ratio of plasma extract:standard. The standard concentration corresponded to the theoretical value of the sample CPE extract using the drug concentration coefficient (sample volume/acetonitrile volume) was corrected by the measured (using a Hamilton syringe) micelle-rich phase volume.

For reproducibility determination two series (over two days) of four plasma samples containing the drugs were extracted and analyzed. The relative standard deviation (RSD%) . was calculated.

To determine LODs plasma was spiked with the standard drugs at four concentrations: 0.125, 0.25, 0.5 and 1 μg mL^{-1} (except paracetamol, which was spiked at 0.25, 0.5, 2, 5 and 10 μg mL^{-1}) and then subjected to CPE-HPLC. The LOD for each drug was calculated as its concentration corresponding to its peak area ≥ 3x baseline noise.

The linear range for each drug was also determined; correlation coefficients are in Table 2.

4. Conclusions

The following conclusions were drawn:

- the CPE technique is satisfactory for plasma sample preparation for HPLC-UV(DAD), drug screening for hydrophobic compounds. Less hydrophobic drugs (*e.g.* paracetamol) may be also determined at sufficiently high concentrations,.

- the drugs tested may be determined at high therapeutic or low toxic plasma concentrations.

- the detection limit for some drugs (amitriptyline, nortriptyline and clomipramine) may be lowered at least twofold if another wavelength (*e.g.* 210 nm) is chosen; however, matrix interference peaks must be considered.

CPE is a good alternative to the routine extraction techniques like LLE or SPE in toxicological drug screening because of its analytical parameters as well as its simplicity, speed, low cost and “green” character.

References

[1] A.C. Moffat, M.D. Osselton, B. Widdop (Eds.), Clarke’s Analysis of Drugs and Poisons, 3rd edition (Pharmaceutical Press, London, 2004)
[2] L. Mercolini, R. Mandrioli, G. Finizio, G. Boncompagni, M.A. Raggi, J. Sep. Sci. 33, 23 (2010)
[3] L. Mercolini, M. Grillo, C. Bartoletti, G. Boncompagni, M.A. Raggi, Anal. Bioanal. Chem. 388, 235 (2007)
[4] G. Zhang, A.V. Terry Jr., M.G. Bartlett, J. Chromatogr. B 856, 20 (2007)
[5] C. Flores-Pérez, J.L. Chávez-Pacheco, B. Ramírez-Mendiola, R. Alemón-Medina, R. García-Álvarez, H. Juárez-Olguín, J. Flores- Pérez, Chromatography 25, 760 (2011)
[6] K. Madej, Trends Anal. Chem. 28, 436 (2009)
[7] Z.-m. Zhou, D.-y. Zhao, J. Wang, W.-j. Zhao, M.-m. Yang, J. Chromatogr. A 30, 1216 (2009)
[8] Ch.Ch. Wang, M.O. Luconi, A.N. Masi, L. Fernández, Talanta 72, 1779 (2007)
[9] H. Wu, G.-Y. Zhao, L.-M. Du, Spectrochim. Acta A 75, 1624 (2010)
[10] H. Filik, İ. Şener, S.D. Cekiç, E. Kiliç, R. Apak, Chem. Pharm. Bull. 54, 891 (2006)
[11] A.B. Tabrizi, Bull. Korean Chem. Soc. 27, 1604 (2006)
[12] H. Zhang, H.-K. Choi, Anal. Bioanal. Chem. 392, 947 (2008)
[13] A. Ohashi, M. Ogiwara, R. Kieda, H. Okada, K. Ohashi, Anal. Sci. 20, 1353 (2004)
[14] M.H. Mashhadizadeh, L. Jafari, J. Iran. Chem. Soc. 7, 678 (2010)
[15] F. Han, R. Yin, X.-l. Shi, Q. Jia, H.-z. Liu, H.-m. Yao, L. Xu, S.-m. Li, J. Chromatogr. B, 868, 64 (2008)
[16] W. Liu, K. Bi, X. Liu, J. Zhao, X. Chen, Chromatographia 69, 837 (2009)
[17] J. Zhou, P. Zeng, H. H. Tu, F. Q. Wang, J. Sep. Sci. 34, 160 (2011)
[18] K. Madej, K. Persona, M. Nizio, Acta Chromatographica 1, 1 (2013)